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BLOCKADE OF HUMAN NEUTROPHIL ACTIVATION BY 2-[2-PROPYL-3-[3-[2-ETHYL-4-(4-FLUOROPHENYL)-5-HYDROXYPHENOXY]PROPOXY]PHENOXY]BENZOIC ACID (LY293111), A NOVEL LEUKOTRIENE B_4 RECEPTOR ANTAGONIST

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Abstract—Leukotriene B_4 (LTB_4), a naturally occurring pro-inflammatory product of arachidonic acid metabolism, has been associated with human inflammatory disease. This study compares the abilities of two LTB_4 receptor antagonists, 2-[2-propyl-3-[3-[2-ethyl-4-(4-fluorophenyl)-5-hydroxyphenoxy]-propoxy]phenoxy]benzoic acid (LY293111) and 7-[3-(4-acetyl-3-methoxy-2-propylphenoxy)-propoxy]-3,4-dihydro-8-propyl-2*H*-1-benzopyran-2-carboxylic acid (SC-41930), to displace LTB_4 binding and their functional blockade of human neutrophil activation. LY293111 inhibited the binding of [^{3}H]LTB_4 with a K_i of 25 nM; SC-41930 displayed a similar potency (K_i = 17 nM). In contrast, LY293111 prevented LTB_4-induced calcium mobilization with an IC_{50} = 20 nM, or 40 times more effectively than SC-41930 (IC_{50} = 808 nM). LY293111 was 300 times more potent than SC-41930 in blocking LTB_4-induced CD11b up-regulation on isolated neutrophils. LY293111 also arrested LTB_4-induced up-regulation of CD11b on neutrophils in whole human blood. LY293111 was not effective in blocking human neutrophil activation responses induced by *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), platelet-activating factor (PAF), human recombinant endothelial interleukin-8 (IL-8) or human recombinant complement component 5a (C5a).

Key words: leukotriene B_4; anti-inflammatory agent; neutrophil; receptor antagonist; flow cytometry

LTB_4† is a pro-inflammatory eicosanoid mediator derived from 5-lipoxygenase-dependent metabolism of arachidonic acid. Elevated levels of LTB_4 or selective pharmacologic blockade of its activity has been reported in a variety of human inflammatory diseases and animal models of such. These include: psoriasis [1], gout [2, 3], colitis [4, 5], allograft rejection [6], periodontal disease [7], endotoxin shock [8, 9] and cystic fibrosis [10–12]. Human neutrophils exhibit specific membrane receptors for LTB_4 [13–15] which, when triggered, affect an array of *in vitro* activation events including chemotaxis, aggregation, superoxide generation, adhesion, mobilization of $[Ca^{2+}]_i$ and up-regulation of cell surface β_2 integrin molecules [16–18]. Several LTB_4 receptor antagonists that either block [^{3}H]LTB_4 binding and/or inhibit subsequent functional events have been identified [16, 18–28].

In this report, we describe the effects of LY293111, a novel diaryl ether carboxylic acid, on displacement of [^{3}H]LTB_4 binding, mobilization of $[Ca^{2+}]_i$, and up-regulation of cell surface CD11b integrin molecules on human neutrophils, either isolated or in whole blood. We compare these results with those obtained using a first generation LTB_4 receptor antagonist, SC-41930. The following data will describe LY293111 as a potent and selective blocker of LTB_4-triggered human neutrophil activation.

MATERIALS AND METHODS

Materials. Low endotoxin, phenol red-free HBSS was purchased from Whittaker Bioproducts, Walkersville, MD. Fatty acid free, low endotoxin BSA (fraction V) was obtained from ICN Biomedicals, Inc., Costa Mesa, CA. Mono-Poly Resolving Medium was acquired from ICN Biomedicals, Inc., Aurora, OH. Indo-1, acetoxymethyl ester was acquired from Molecular Probes, Inc., Eugene, OR. Anti-human CD11b-fluorescein conjugate (Mo-1-FITC) was obtained from the Coulter Corp., Hialeah, FL. IL-8 was purchased from Biosource International, Inc., Westlake Village, CA. PAF, fMLP and C5a were all purchased from the Sigma Chemical Co., St. Louis, MO. Paraformaldehyde, EM grade, was obtained from Polysciences, Inc.,

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† Abbreviations: LTB_4, leukotriene B_4; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; C5a, human recombinant complement component 5a; PAF, platelet-activating factor; IL-8, human recombinant endothelial interleukin-8; $[Ca^{2+}]_i$, intracellular divalent calcium; LY293111, 2-[2-propyl-3-[3-[2-ethyl-4-(4-fluorophenyl)-5-hydroxyphenoxy]propoxy]phenoxy]benzoic acid; SC-41930, 7-[3-(4-acetyl-3-methoxy-2-propylphenoxy)-propoxy]-3,4-dihydro-8-propyl-2*H*-1-benzopyran-2-carboxylic acid; HBSS-BSA, Hanks' balanced salt solution containing 0.1% (w/v) bovine serum albumin; and MFI, mean fluorescence intensity.

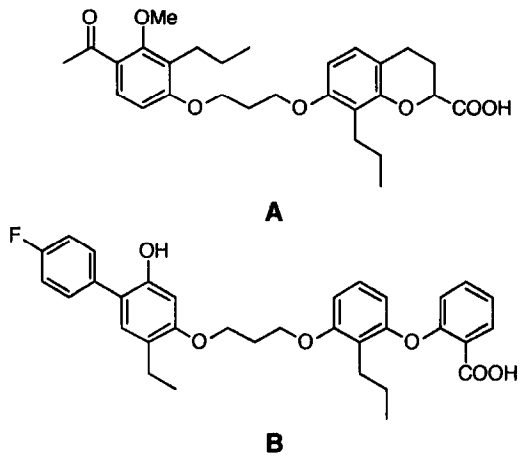

Fig. 1. Structural formulas of the two leukotriene B_4 receptor antagonists used in this study: (A) SC-41930 7-[3-(4-acetyl-3-methoxy-2-propylphenoxy)-propoxy]-3,4-dihydro-8-propyl-2*H*-1-benzopyran-2-carboxylic acid, and (B) LY293111 2-[2-propyl-3-[3-[2-ethyl-4-(4-fluorophenyl) - 5 - hydroxyphenoxy]propoxy]phenoxy]-benzoic acid.

Warrington, PA. FACS® Lysing Solution was purchased from Becton Dickinson Immunocytometry Systems, San Jose, CA. LTB_4 was obtained by total synthesis and purified as previously reported [29, 30] at the Lilly Research Laboratories. [^{3}H]LTB_4 (150–220 Ci/mmol) was purchased from NEN Research Products, Wilmington, DE. Deep well polypropylene 96-well micro-assay plates were purchased from Beckman Instruments, Fullerton, CA. Sodium salts of compounds LY293111 and SC-41930 were synthesized at Lilly Research Laboratories (Fig. 1). All compounds, reagents and cells were dissolved, washed or suspended for assay in HBSS-BSA.

Neutrophil preparation. Human blood was collected from healthy volunteers by venipuncture using EDTA (1.5 mg/mL, final concentration) as an anticoagulant. Polymorphonuclear leukocytes (neutrophils) were isolated by discontinuous gradient separation over Mono-Poly Resolving Medium [31, 32]. The resulting cell pellet was cleared of erythrocytes by hypotonic lysis. The neutrophil preparation (> 92% purity and > 98% viability) was washed twice and suspended in HBSS-BSA. For each experiment, neutrophils were isolated from a single donor.

[^{3}H]*LTB_4 displacement.* A micro-assay modification of a published technique [16, 33] was utilized to test the ability of the experimental compounds to displace [^{3}H]LTB_4 binding to human neutrophils. Added to deep well micro-assay plates were 55 μL of diluted compound and 250 μL of [^{3}H]LTB_4 (0.35 nM, final concentration), and binding was initiated upon the addition of 250 μL of isolated human neutrophils (6×10^6 cells/mL stock). The plates were incubated for 20 min at 4°. The resultant mixtures were harvested on a Skatron (Wallac, Inc., Gaithersburg, MD) multiwell harvester on 1.5 μm glass fiber filter pads (Wallac). Radioactivity for each experimental sample was determined using a Wallac Betaplate® beta ray scintillation counter. The K_i for each compound was extrapolated from the observed data using an appropriate algorithm [34, 35].

Calcium mobilization. Use of flow cytometry for measuring $[Ca^{2+}]_i$ mobilization has been described previously [18, 36, 37]. Briefly, neutrophils (5×10^7) were suspended in 5 mL of Indo-1 (10 μM) and allowed to rock at 37° for 45 min. Cells were washed twice and suspended at 1×10^6/mL in HBSS-BSA. To 0.98 mL of cell suspension, 10 μL of putative antagonist was added, mixed and allowed to equilibrate for 3 min at room temperature. The sample was then introduced into an Epics V flow cytometer (Coulter Corp.), which was used with its laser tuned to emit UV light (350–380 nm). The emitted cell-associated fluorescence was captured

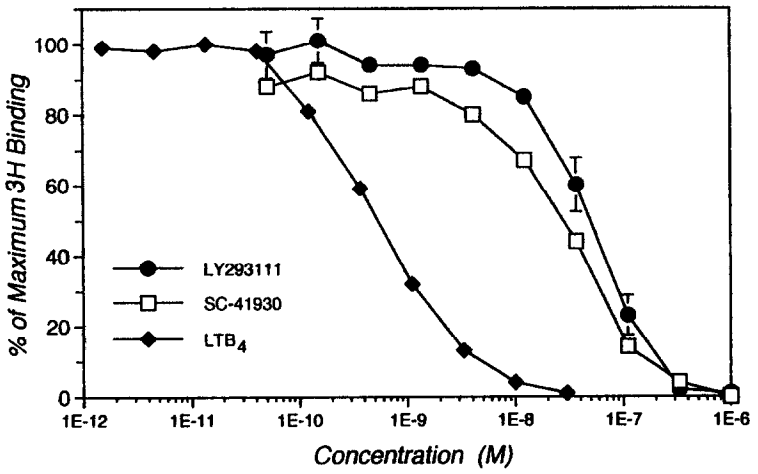

Fig. 2. Displacement of [^{3}H]LTB_4 binding to human neutrophils by LTB_4, SC-41930 and LY293111. Isolated human neutrophils, experimental compounds and ~ 75,000 dpm of [^{3}H]LTB_4 (0.35 nM, final concentration) were reacted together for 20 min at 4°. The samples were then harvested and analyzed for bound [^{3}H]. Data represent the mean ± SEM percentage of maximum [^{3}H] bound (ordinate) for each compound concentration (e.g. 1E-11 = 1×10^{-11} molar) over three separate experiments.

through a special "Indo-1 filter set" (Omega Optical, Inc., Brattleboro, VT). For each sample, the ratio of 405:485 nm light emission was derived through processing of analog circuitry and captured in light scatter-gated two parameter histograms of time versus fluorescence ratio (indicating relative $[Ca^{2+}]_i$). All samples were run at room temperature and at a maximum rate. Data were collected for each antagonist preincubated sample for at least 12 sec before interruption for the addition of 10 μL of agonist. Total analysis time per sample was approximately 6 min. Two-parameter histograms for each treatment condition were analyzed for maximum percentage of responding cells using the "CaFlux" software (Peanut Jelly Software, Denver, CO). The inhibitory activity for each experimental condition was computed as follows:

% Inhibition of $[Ca^{2+}]_i$ release

$$= 100 \times \left\{1 - \left[\frac{(\%\text{ Cells releasing } [Ca^{2+}]_i \text{ with antagonist}) - (\text{Background \% cells releasing } [Ca^{2+}]_i)}{(\text{Maximum \% cells releasing } [Ca^{2+}]_i) - (\text{Background \% cells releasing } [Ca^{2+}]_i)}\right]\right\}$$

Isolated neutrophil CD11b up-regulation. Up-regulation of CD11b on activated human neutrophils has been utilized before to study the effects of LTB_4 receptor antagonists [18]. Briefly, for each reaction mixture, 0.9 mL of HBSS-BSA containing 1×10^6 isolated human neutrophils was preincubated with 10 μL of putative antagonist for 15 min at room temperature. To these suspensions, 100 μL of agonist was added, mixed and allowed to incubate in a 37° water bath for 30 min. The samples were then cooled to 4°, washed, and incubated with 100 μL of anti-CD11b-FITC (50 μg/mL stock) for 30 min at 4°. Cells were then washed in HBSS-BSA and fixed with paraformaldehyde (1% in phosphate-buffered saline) before flow cytometric analysis. Fixed samples were gate-analyzed for green fluorescence associated with the forward versus the 90° neutrophil light scatter region, using an Epics XL flow cytometer (Coulter Corp.), as previously described [18]. The MFI for each sample was computed using the on board software package of the cytometer. The inhibitory activity for each experimental condition was computed as follows:

% Inhibition of CD11b up-regulation

$$= 100 \times \left\{1 - \left[\frac{(\text{MFI of cells treated with antagonist}) - (\text{MFI of cells without agonist})}{(\text{Maximum MFI of cells with agonist}) - (\text{MFI of cells without agonist})}\right]\right\}$$

Whole blood CD11b up-regulation. Ninety microliters of whole, EDTA treated blood was dispensed carefully into 12 × 75 mm polypropylene tubes. Directly into this sample, 10 μL of putative antagonist was pipetted, vortexed and incubated for 15 min at room temperature. Cell activation was initiated with the addition of 10 μL of agonist. The resulting mixtures were incubated for an additional 30 min at 37°. The samples were cooled to 4°, and concentrated anti-CD11b-FITC (10 μL of a 500 μg/mL solution) was added, mixed and incubated with the blood mixtures for 30 min at 4°. Red blood cells were then lysed using FACS® Lysing Solution, per the manufacturer's instructions. The samples were then washed and stored fixed (in 1% paraformaldehyde) at 4° and flow cytometrically analyzed as described above for isolated neutrophils.

RESULTS

$[^3H]LTB_4$ displacement. The ability of SC41930 and LY293111 to competitively inhibit the binding of $[^3H]LTB_4$ to isolated human neutrophils is depicted in Fig. 2. The IC_{50} values (± SEM) for LY293111 and SC-41930 were 42.5 ± 5.9 and 28.1 ± 7.4 nM, respectively. From these data, and with the incorporation of an empirically determined dissociation constant (K_d) for LTB_4 of 0.45 nM, K_i values were determined for LY293111 and SC-41930: 25.3 ± 3.5 and 16.5 ± 4.2 nM, respectively (Table 1). Thus, LY293111 and SC-41930 displayed a nearly equivalent ability to block the binding of $[^3H]LTB_4$ to isolated human neutrophils.

Inhibition of calcium mobilization. Comparison of LY293111 and SC-41930 abilities to block LTB_4 induced release of $[Ca^{2+}]_i$ in isolated human neutrophils is depicted in Fig. 3. These data represent an inhibition of the *fraction of cells* releasing $[Ca^{2+}]_i$ rather than the absolute amount of Ca^{2+} released per cell or in total. This approach obviates the requirement for a strict spectrophotometric $[Ca^{2+}]_i$ calibration and has been shown previously to be useful for such determinations [18]. From these data, SC-41930 affected a mean IC_{50} of 808 nM, while that of LY293111 was 20.7 nM (Table 1), or approximately 40 times more potent.

Inhibition of CD11b up-regulation on isolated human neutrophils. Preincubation of isolated human neutrophils with SC-41930 and LY293111 elicited a concentration-dependent inhibition of LTB_4-induced CD11b up-regulation, as depicted in Fig. 4. SC-41930 blocked this effect with an IC_{50} of 1.45 μM, while LY293111 was 370 times more potent at 3.94 nM (Table 1).

Inhibition of CD11b up-regulation on neutrophils in whole blood. Incubation of whole, human blood with LTB_4 induced a concentration-dependent up-regulation of CD11b on neutrophil surfaces, as displayed in Fig. 5. In this whole blood system, a plateau of CD11b up-regulation commenced at 10 nM LTB_4, or approximately 10 times that required for CD11b up-regulation of isolated neutrophils [18]. When whole, human blood was preincubated with either SC-41930 or LY293111, followed by stimulation with 10 nM LTB_4, a concentration-dependent inhibition of CD11b up-regulation on

Table 1. Effects of LY293111 and SC-41930 on [^{3}H]LTB$_4$ displacement and LTB$_4$-induced activation of human neutrophils

Human neutrophil assay	LY293111* (nM)	SC-41930† (nM)
[^{3}H]LTB$_4$ displacement	25.3‡	16.5‡
$[Ca^{2+}]_i$ mobilization	20.7§	808.0§
CD11b up-regulation (isolated cells)	3.9§	1450.0§
CD11b up-regulation (whole blood)	362.0§	30100.0§

* (2 - [2 - Propyl - 3 - [3 - [2 - ethyl - 4 - (4 - fluorophenyl) - 5 - hydroxyphenoxy]-propoxy]-phenoxy]benzoic acid) sodium.

† (7-[3-(4-Acetyl-3-methoxy-2-propylphenoxy)-propoxy]-3,4-dihydro-8-propyl-2*H*-1-benzopyran-2-carboxylic acid) sodium.

‡ K_i (affinity constant).

§ IC_{50} (inhibitory concentration, 50%).

human neutrophils was produced, as displayed in Fig. 6. From these data, SC-41930 was computed to have an IC_{50} of 30.1 ± 0.09 μM, while LY293111 was approximately 80 times more potent with an IC_{50} of 0.362 ± 0.09 μM (Table 1).

Selectivity of LY293111 on LTB$_4$-mediated responses. The selectivity of the effects of LY293111 on human neutrophils was tested in other experiments. In data summarized in Fig. 7, isolated human neutrophils were preincubated with increasing concentrations of LY293111 followed by fMLP (10 nM) challenge. In this system, LY293111 exhibited an IC_{50} of 38 ± 2.6 μM or ~ 10,000-fold less active than when using LTB$_4$ as an agonist. In addition, as depicted in the representative results depicted in Fig. 8, LY293111 was ineffective in blocking C5a-, PAF- or IL-8-induced $[Ca^{2+}]_i$ mobilization of isolated, human neutrophils at concentrations up to 10 μM (~ 500 times the LTB$_4$-induced $[Ca^{2+}]_i$ mobilization IC_{50}).

DISCUSSION

In this investigation, we compared the effects of two LTB$_4$ receptor antagonists, SC-41930 and LY293111, on human neutrophils *in vitro*. We measured the ability of each compound to block the binding of [^{3}H]LTB$_4$ and their ability to prevent LTB$_4$-induced CD11b up-regulation and $[Ca^{2+}]_i$ mobilization in isolated human neutrophils. We also described LTB$_4$-stimulated CD11b up-regulation on human neutrophils in whole human blood and the ability of LY293111 and SC-41930 to block this phenomenon. Finally, we describe the selectivity of LY293111 toward blocking LTB$_4$-induced activation.

The data (Table 1) reveal that the novel LTB$_4$ receptor antagonist LY293111 was not substantially different from SC-41930 in its ability to block the binding of [^{3}H]LTB$_4$. In marked contrast, LY293111 was substantially more potent than SC-41930 in blocking functional activation of neutrophils

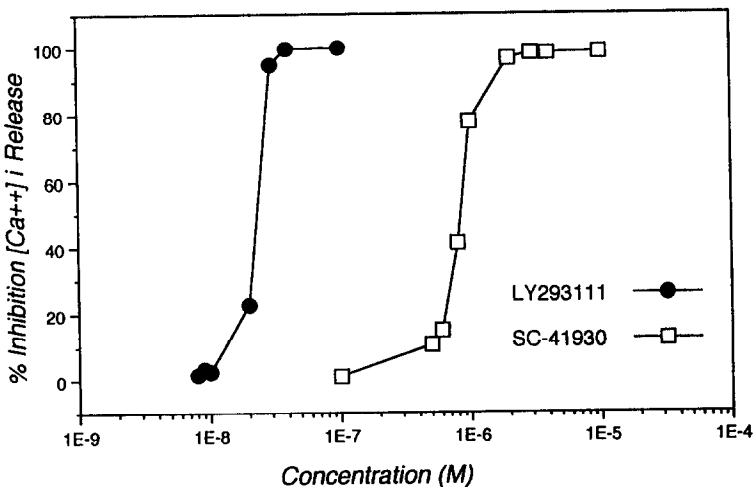

Fig. 3. Inhibition of LTB$_4$-induced $[Ca^{2+}]_i$ mobilization in human neutrophils by LY293111 and SC-41930. Isolated human neutrophils were loaded with Indo-1 calcium detection dye, preincubated for 3 min with various concentrations of either LY293111 or SC-41930 and flow cytometrically analyzed over time for $[Ca^{2+}]_i$. After steady-state measurements, LTB$_4$ was added and the percentage of cells releasing $[Ca^{2+}]_i$ was determined. The percent inhibition from control is plotted for each compound/concentration treatment. Data points represent the mean activity from 3 experiments with variation (SEM) of less than 10%.

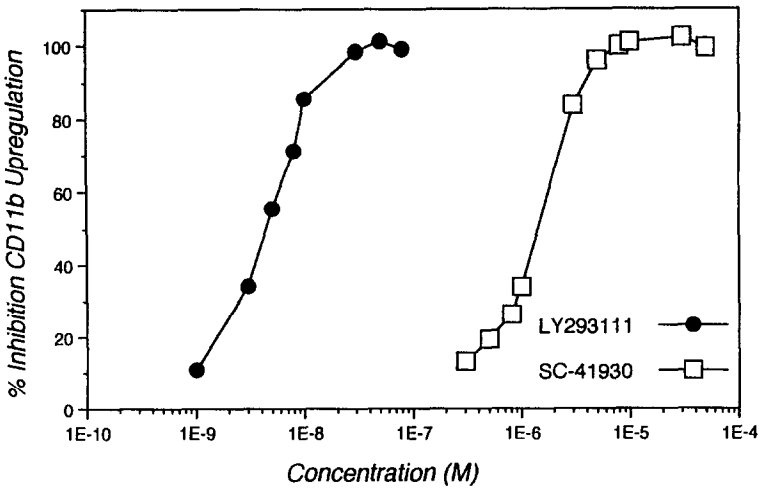

Fig. 4. Effects of LY293111 and SC-41930 on LTB_4-induced up-regulation of CD11b integrin expression on isolated human neutrophils. Isolated human neutrophils were preincubated with LY293111 or SC-41930 for 15 min at room temperature followed by incubation with LTB_4 (1 nM) for 30 min at 37°. Cells were cooled, washed and reacted with anti-CD11b-FITC. The cells were then fixed and analyzed for specific CD11b integrin expression using flow cytometry. The inhibition from control is plotted for each compound/concentration treatment. Data points represent the mean activity from 3 experiments with variation (SEM) of less than 10%.

subsequently stimulated with LTB_4. LY293111 was 40 times more effective than SC-41930 in blocking LTB_4-induced $[Ca^{2+}]_i$ mobilization and likewise more potent than SC-41930 in blocking LTB_4-induced CD11b up-regulation on isolated and whole blood neutrophils (400 and 80 times, respectively). Also, our data strongly indicate that this blockade of neutrophil activation by LY293111 is not due to a generalized physiologic toxicity in these cells. First, no changes in cell viability were noted in any experiment where LY293111 was used (data not shown). Furthermore, LY293111 did not block neutrophil $[Ca^{2+}]_i$ mobilization stimulated by IL-8, PAF or C5a at 500 times its LTB_4-induced IC_{50} and required ~ 10,000 times its LTB_4-associated IC_{50} to block fMLP-induced CD11b up-regulation (Figs. 7 and 8).

The basis of the enhanced functional potentcy of LY293111 is not understood. Explanations for this phenomenon can be hypothesized. For example, it is possible that LY293111 has a distinctive binding relationship to the high-affinity state of the LTB_4 receptor. Such LTB_4 receptor states have been described by others [13, 14, 27, 38–40]. Indeed, both $[Ca^{2+}]_i$ mobilization and CD11b-dependent adhesion of neutrophil events are associated with high-affinity states of the LTB_4 receptor [38]. The superior potency of LY293111 over SC-41930 in inhibiting

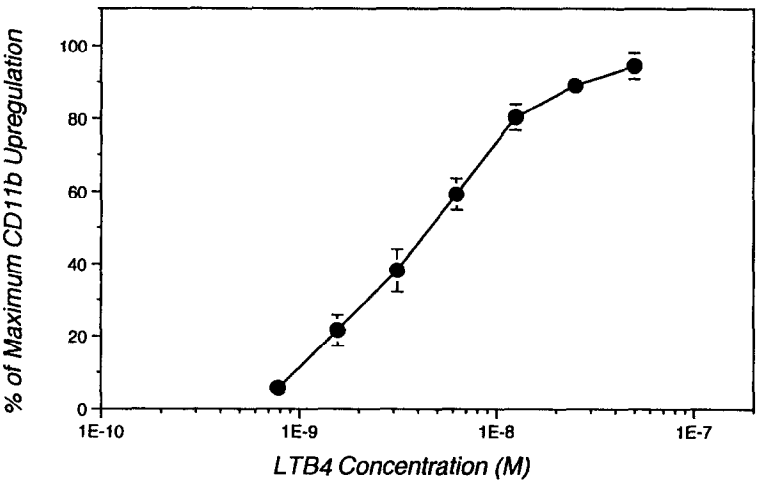

Fig. 5. Up-regulation of CD11b on human neutrophils residing in whole human blood. Blood from healthy volunteers was drawn in EDTA anti-coagulant, mixed with increasing concentrations of LTB_4, and incubated for 30 min at 37°. Cells were cooled to 4° and reacted for 30 min with anti-CD11b-FITC. The samples were then RBC-lysed, fixed and analyzed for CD11b expression using flow cytometry. Each data point represents the mean ± SEM of 3 volunteers.

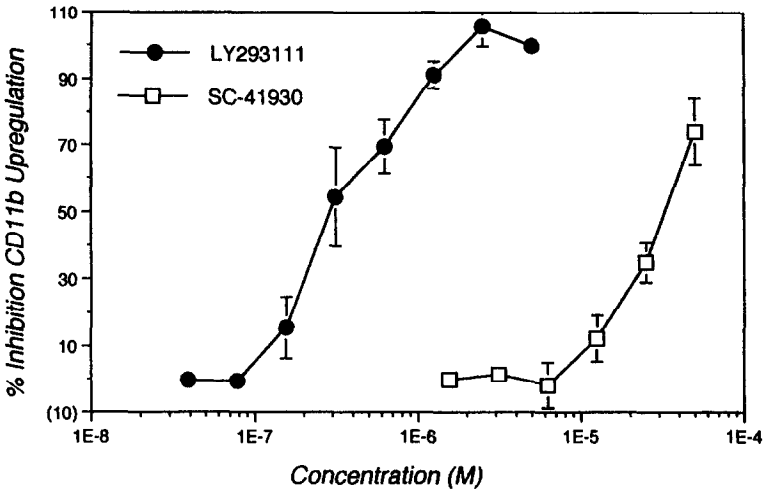

Fig. 6. Effects of LY293111 and SC-41930 on LTB_4-induced up-regulation of CD11b integrin expression of human neutrophils residing in whole blood. Whole blood, drawn in EDTA, was preincubated with LY293111 and SC-41930 for 15 min at room temperature followed by incubation with LTB_4 (10 nM) for an additional 30 min at 37°. Cells were washed, cooled and reacted with anti-CD11b-FITC for 30 min at 4°. These samples were then RBC-lysed, fixed and analyzed for specific CD11b integrin expression using flow cytometry. Each data point represents the mean (± SEM) response of 4 volunteers.

these two functions without an increased ability to block total [^{3}H]LTB_4 binding may be due to some distinctive relationship that LY293111 has for the high-affinity LTB_4 receptor state.

In this report, we also describe measurement of LTB_4-induced CD11b up-regulation on human neutrophils in whole blood. This up-regulation was concentration dependent and highly reproducible (Fig. 5). We were also able to detect and quantitate the ability of LY293111 to block this phenomenon (Fig. 6). Although its potency was somewhat attenuated (Table 1), the ability of LY293111 to block LTB_4-induced activation while present in the blood milieu is an important tool for the clinical development of LY293111 as an anti-inflammatory agent. Blood from volunteers receiving doses of

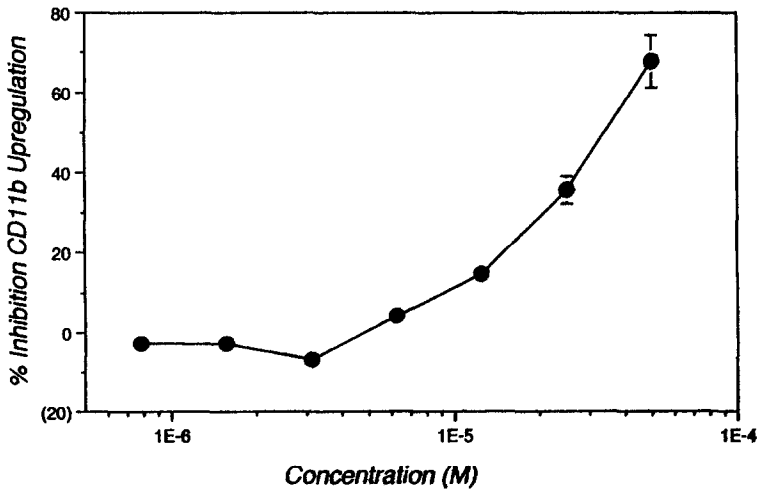

Fig. 7. Effect of LY293111 on CD11b integrin expression stimulated by fMLP. Isolated human neutrophils were preincubated with LY293111 for 15 min at room temperature followed by incubation with fMLP(10 nM) for 30 min at 37°. Cells were cooled, washed and reacted with anti-CD11b-FITC. The cells were then fixed and analyzed for specific CD11b integrin expression using flow cytometry. The percent inhibition of maximum CD11b up-regulation is plotted for each LY293111 concentration. Data points represent the mean (± SEM) from 3 separate experiments.

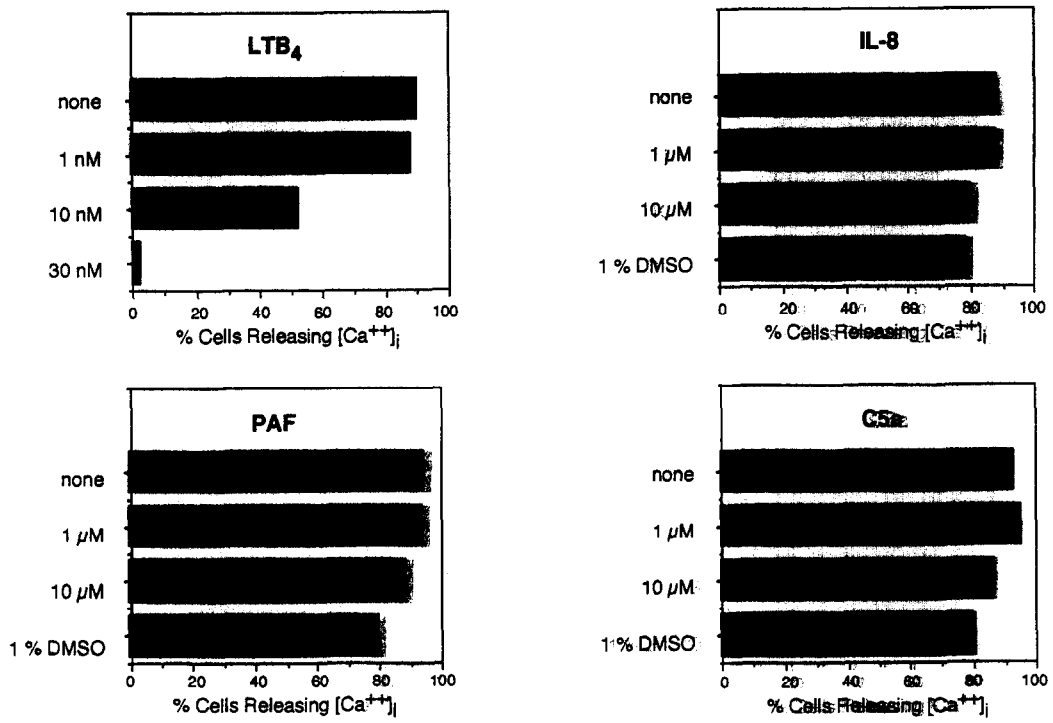

Fig. 8. Selectivity of LY293111 on inhibiting $[Ca^{2+}]_i$ mobilization of human neutrophils. Isolated human neutrophils were loaded with Indo-1 calcium detection dye, preincubated for 3 min with various concentrations of LY293111, and flow cytometrically analyzed over time for $[Ca^{2+}]_i$. After steady-state measurements, either LTB_4, IL-8, PAF or C5a was added in optimal concentrations, and the percentage of cells releasing $[Ca^{2+}]_i$ was determined. Data are taken from a representative experiment.

LY293111 can be challenged *ex vivo* with LTB_4 and CD11b up-regulation measured. Hence, the pharmacologic efficacy and pharmacodynamic profile of LY293111 can be determined independently from its therapeutic effects on inflammatory disease in humans.

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